Enteric Nervous System Transduction of Different AAV Serotypes Following Intravenous Injection

Undergraduate Research Thesis

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By

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Abstract:

Functional gastrointestinal disorders (FGID) affect 1 in 4 Americans and are likely caused by dysfunction of the enteric nervous system (ENS). The chronic nature of FGID suggests gene therapy is a promising form of treatment and can be used to treat diseases such as Hirschrprung's disease. In this study, we characterized transduction efficiency and distribution in the mouse myenteric plexus following intravenous (IV) injection of adeno-associated viral vectors (AAV) expressing green fluorescent protein (GFP). Neonatal (P1) mice were injected with AAV9 in the temporal vein and juvenile mice (P21) were injected in the tail vein. Mice were euthanized at 60-90 days of age, and the myenteric plexus was examined. Immunohistochemical labeling of the myenteric plexus revealed GFP presence along the entire GI tract in injected mice. Neuronal transduction ranged from 21-57% depending on vector dose and GI region analyzed. GFP co-localized with interneurons, excitatory and sensory myenteric neurons while GFP expression was absent in VIP, nNOS and glial cells. Neonate IV injections with AAV1, 5, and 6 containing GFP produced little to no transgene expression in the myenteric plexus. However, AAV8 showed significantly higher neuronal transduction compared to AAV9 injected mice. Collectively, these data demonstrate that enteric neurons are efficiently transduced by AAV8 and AAV9 following IV delivery. Thus, AAV8 and AAV9 can be used as possible treatments for ENS dysfunction by the expression of a transgene.

Introduction:

Use of Adeno-associated Virus in Gene Therapy

Adeno-associated virus (AAV) vectors have been shown to be promising in gene therapy applications and are currently permitted for many clinical trails. In order for gene therapy to be successful, a viral vector needs to be safe, achieve efficient infection of target tissue, and establish either long-term or short-term gene expression. AAV is a non-pathogenic single-stranded DNA parovirus with multiple serotypes, which increases its potential as a delivery vehicle for gene therapy application. There are three main components of the AAV genome, namely its inverted terminal repeats (ITR), rep gene, and cap genes (Daya and Berns, 2008). The rep gene allows for replication and the cap gene encapsidates the viral genome during replication. ITRs play a key role in DNA replication of the viral genome and integration into host cells. When engineering a recombinant AAV (rAAV) the rep and cap genes are removed leaving only the ITRs (Goncalves, 2005). An expression cassette including a promoter, transgene and poly A is then inserted in between the ITRs, allowing the transgene to be produced by an infected cell. The newly engineered viral genome is placed in the capsid of the AAV viral particle. This capsid is an outside protein coating, which will determine the serotype of the AAV vector and allows the virus to enter cells via different receptors (Choi et al., 2005, Wu et al., 2006).

It has recently been discovered that some AAV serotypes cross the blood-brain barrier (BBB) and can result in therapeutic benefit in animal models of neurological disease (Foust et al., 2010, 2013; Fu et al., 2011; Ahmed et al., 2013; Garg et al., 2013; Haurigot et al., 2013). AAV serotype 9 (AAV9) has been well characterized within the central nervous system (CNS) and has shown to have robust transduction in the CNS of mice, rats, cats and non-human

primates (Duque et al., 2009; Tatom et al., 2009; Bevan et al., 2011). Additionally, serotypes 1, 6 and 7, along with other serotypes, cross the BBB and produce transgene expression at varying levels within the CNS (Zang et al., 2011). AAV's ability to transduce central nervous tissue following systemic deliver suggests that the peripheral nervous systems (PNS) is likely transduced as well. Gene delivery to the PNS and CNS can be beneficial for treating global neurological diseases.

The Enteric Nervous System

The enteric nervous system (ENS) is located in the gut and operates independently of the CNS to coordinate the complex actions within the gastrointestinal (GI) tract. The ENS contains as many neurons as the spinal cord with neurotransmitter diversity comparable to the CNS (Gershon and Ratcliffe, 2004). These neurons, along with enteric glia, reside in ganglia that form neural plexuses within the gut. The ENS is divided into two nerve plexuses, namely the submucosal plexus and the myenteric plexus. The submucosal plexus is located between the mucosal and circular muscle layers and is responsible for the chemoreception, mechanoreception, and secretory functions within the gut as well as some aspects of motility. The myenteric plexus is home to enteric motor neurons and is located between the circular and longitudinal smooth muscle layers. The myenteric plexus is responsible for motility as a result of neuromuscular transmission to smooth muscle, resulting in peristalsis. Peristalsis is the coordinated contraction and relaxation of smooth muscle that propagates in a wave-like action to move contents through the gut. Impaired neuromuscular transmission results in severe GI dysfunction. Apart from enteric neurons, other cell types are also important for proper GI function.. For example, enteric glia are essential for maintaining homeostasis and regulating

neural circuit activity (Gulbransen and Sharkey, 2012) and interstitial cells of Cajal are pacemakers in the gut wall and are thought to mediate neurotransmission between enteric motor neurons and smooth muscle (Ward and Sanders, 2006).

Methods:

Intravenous Injection of AAV.

All studies used self-complementary AAV with a chicken β actin promoter expressing green fluorescent protein (scAAV-CB-GFP). *Postnatal day 1* (P1) neonatal pups were injected with 5e10 vg/ml of AAV serotypes 1, 5, 6, 8, or 9 expressing green fluorescent protein (GFP). Pups were anesthetized on wet ice and injected in the temporal face vein under a dissecting microscope. Pups were warmed and returned to their home cage with the dam. Mice were euthanized by transcardial perfusion 30-60 days post-injection for myenteric dissection. When comparing age of administration, neonatal pups were injected at a dose of 1e11 vg/ml. *Juvenile mice* (P21) were injected in the tail vein while restrained in an illuminated tail vein injection platform. Mice were injected with AAV9 expressing GFP for a final dose of $2x10^{12}$ vg and returned to their home cages. Mice were euthanized by transcardial perfusion 30-60 days post-injection for myenteric dissection.

Dissection of the myenteric plexus.

Entire gastrointestinal tracts (stomach, duodenum, jejunum, ileum, cecum and colon) were removed from euthanized mice after a saline rinse. Each segment of the GI tract was then separated, opened, and pinned flat in a dissection dish. Tissues were fixed overnight at 4° in Zamboni's fixative (paraformaldehyde and picric acid). The next day, tissues were rinsed of

fixative and the mucosal, submucosal, and circular muscle layers were removed to expose the myenteric plexus on the longitudinal muscle.

Immunohistochemistry.

Brain and Spinal Cord: CNS tissues were post-fixed in 4% paraformaldehyde, and brains were further dehydrated in 30% sucrose solution. Brains and spinal cords were sliced at 40μm, and incubated in blocking solution (10% normal donkey serum, 1% Trition-X 100 in PBS) for two hours at room temperature. Sections were then incubated in primary antibody, chicken anti-GFP (1:2000), mouse anti-NeuN (1:200, neurons), or rabbit anti-GFAP (glial fibrillary acidic protein, 1:500), overnight at room temperature. The next day sections were incubated with the appropriate fluorescent secondary (1:500) for two hours at room temperature, were mounted on slides and coverslipped.

Myenteric plexus chemical coding: GI tissue was blocked for 1 hour in blocking solution (10% normal donkey serum, 0.5% Trition-X 100 in PBS) followed by primary antibody made in 0.5% Triton-X 100 PBS at 4° overnight. For initial quanitifcation, primary antibodies included chicken anti-GFP (1:200) and mouse anti-HuD (1:50) to identify transduced neurons. The next day, tissue was incubated with the appropriate fluorescent secondary antibodies (1:200) at room temperature for 2 hours. Tissue was then rinsed, placed on slides and coverslipped. For further chemical coding tissues were incubated in appropriate primary antibody overnight at 4°C against green fluorescent protein (GFP; 1:500), HuD (1:25), S100 (1:200), choline acetyltransferase (ChAT; 1:50), vasoactive intestinal peptide (VIP; 1:200), neuronal nitric oxide synthase (nNOS; 1:200), calretinin (1:200), or calbindin (1:200). The next day, tissues were rinsed 4 times for 10 minutes in PBS, and then incubated in appropriate secondary antibodies at 1:200 for 2 hours at

room temperature. Antibody dilutent was 3% NDS, 0.5% Triton-X 100 in PBS. Tissues were then rinsed and coverslipped with 2.5% PVA/DABCO. Fluorescent images were captured on an Olympus confocal imaging system.

Quantification of AAV9 transduced neurons.

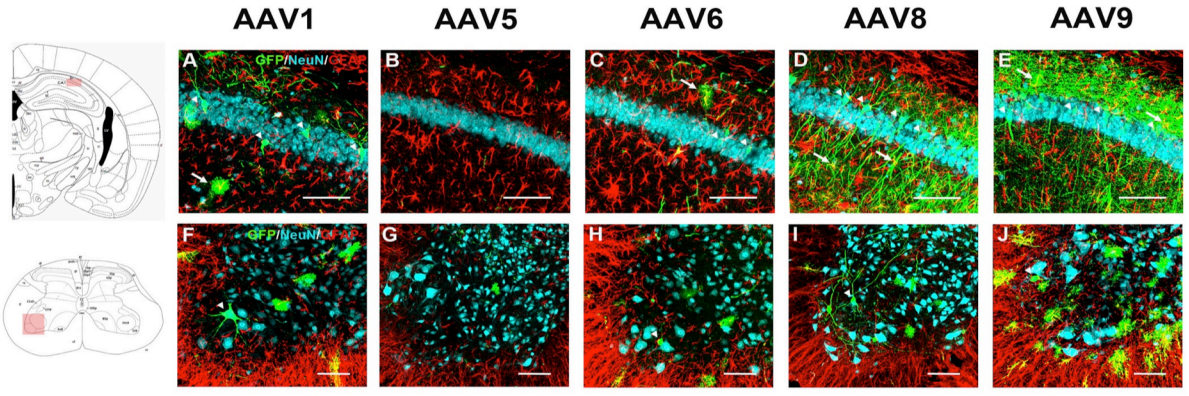
HuD positive neurons (red) and GFP positive neurons (green) were counted under the microscope at 20x. GFP positive neurons represented the successfully transduced neurons, and percentages of transduction were calculated from the total amount of neurons present.

Results:

Transduction of the CNS

To investigate AAV serotype transduction of the CNS, neonatal (P1) mice were injected with 5e10 vg/ml of AAV serotypes 1, 5, 6, 8, or 9 expressing green fluorescent protein (GFP).

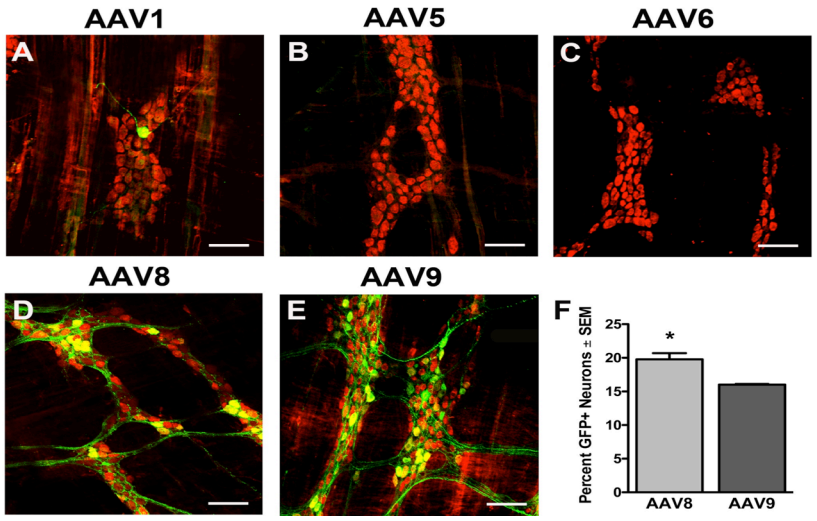
Figure 1.



AAV Transduction in the Brain and Spinal Cord following intravenous injection. GFP immunofluorescence was detected in neurons (NeuN, cyan) and astrocytes (glial fibrillary acidic protein (GFAP), red) in the brains and spinal cords of AAV1 (**A,F**), AAV6 (**C,H**), AAV8 (**D,I**), and AAV9 (**E,J**) intravenously injected mice. No CNS transduction occurred in AAV5 (**B,G**) injected animals. Arrowheads indicate transduced neurons (co-labeling with NeuN) and arrows indicated transduced astrocytes (co-labeled with GFAP). Scale bars are 100μm.

Mice were euthanized 30-60 days post-injection and the brain and spinal cord slices were immunolabeled for GFP, NeuN and GFAP. Immunolabeling revealed CNS transduction in AAV1, AAV6, AAV8 and AAV9, injected mice with no transduction occurring in AAV5 injected mice (**Figure 1**). The most robust CNS transduction was observed following AAV8 and AAV9 injection.

Figure 2.



Transduction efficiency in the myenteric plexus is dependent on AAV serotype. Neonatal mice were intravenously injected with AAV1 (**A**), AAV5 (**B**), AAV6 (**C**), AAV8 (**D**), or AAV9 expressing GFP (**E**) (final dose $5x10^{10}$ GC/ml). Green fluorescent protein (GFP, green) immunofluorescence in the myenteric plexus of the colon reveled little to no GFP expression in neurons (HuD, red) following AAV1, AAV5, and AAV6 intravenous injection. Robust GFP expression was detected in neurons in AAV8 (**D**) and AAV9 (**E**) injected mice. Total counts of neurons in the myenteric plexus of the colon revealed that 19.7% ± 0.9 or 16.0% ± 0.2 of neurons were transduced in AAV8 and AAV9 injected mice, respectively (**F,** p <0.001). Scale bars are 100µm.

Transduction within the Myenteric Plexus

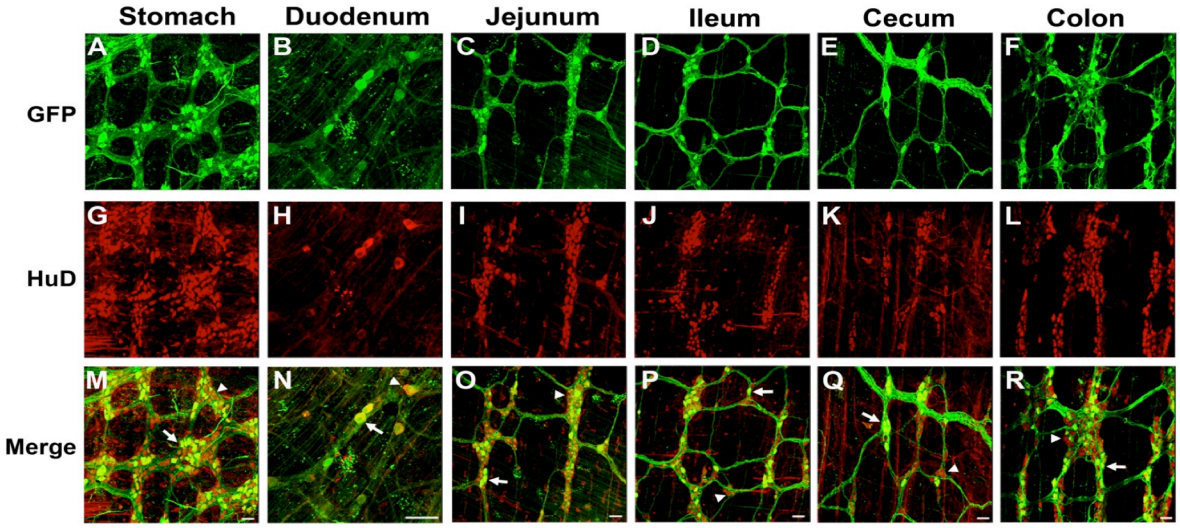
To investigate if various AAV serotypes could also transduce the myenteric plexus, neonatal (P1) mice were injected as described above. Mice were euthanized 30-60 days post-injection and myenteric plexus was immunolabed for GFP and HuD. Immunolabeling revealed little to no transduction occurred following AAV1, AAV5 and AAV6 intravenous injection. However, robust transduction was seen in AAV8 and AAV9 injected mice (**Figure 2**). GFP

positive myenteric neurons in AAV8 and AAV9 injected mice were quantified. In AAV8 injected mice, a total of 3029 HuD positive and 599 GFP positive neurons were counted from 91 ganglia. In AAV9 injected mice, a total of 3366 HuD positive and 539 GFP positive neurons were counted from 90 ganglia. A t-test revealed that transduction in AAV8 injected mice was significantly higher than that of AAV9 injected mice [$t_{(178)}$= 4.11, $p < 0.001$]. An average of 20.6 ± 1% of neurons were transduced in AAV8 injected mice compared to that of 15.8 ± 1% in AAV9 injected mice.

AAV9 transduction of the myenteric plexus

It has previously been reported that age of AAV9 administration can have a bias for cell type transduction in the CNS (Foust et al. 2009). Intravenous injection of AAV9 in neonatal mice preferentially targets neurons where as astrocytes are targeted in juvenile injected animals.

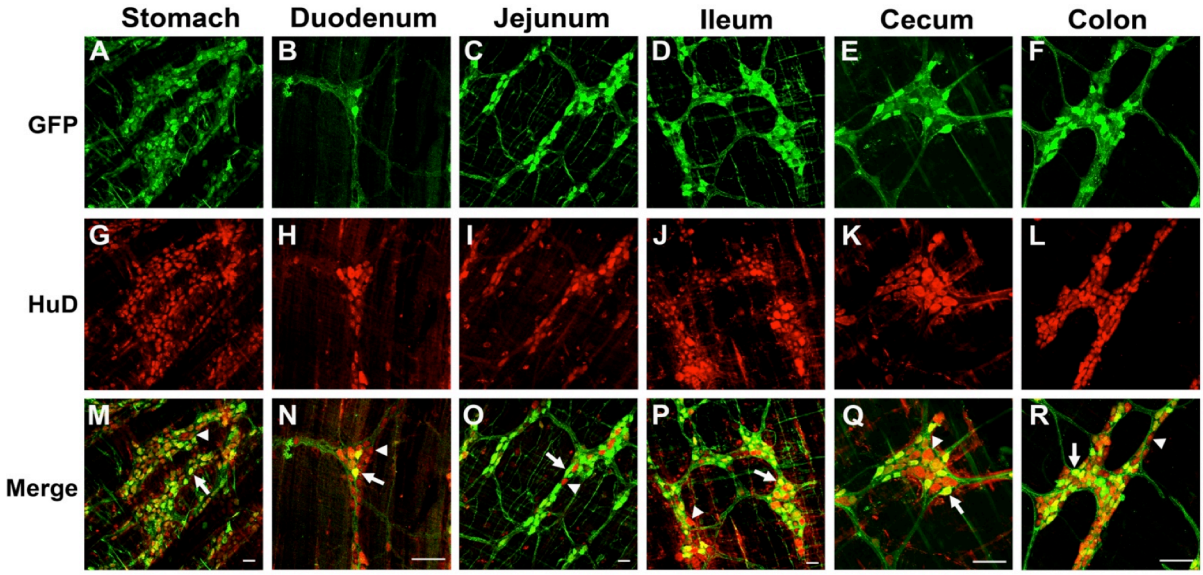
Figure 3.



Green fluorescent protein (GFP) expression in the myenteric plexus following intravenous injection of AAV9 expressing GFP into neonatal mice. Intravenous injection of AAV9 into P1 mice resulted in GFP (green, A-F) expression in neurons (HuD, red, G-L) in the stomach (A, G, M), duodenum (B, H, N), jejunum (C, I, O), ileum (D, J, P), cecum (E, K, Q), and in the colon (F, L, R). Arrows in merged images (M-R) point to GFP expressing neurons. Arrowheads identify untransduced neurons. Scale bars are 100μm.

To determine if the same transduction bias occurred in the myenteric plexus, AAV9 was injected in neonatal (P1) and juvenile (P21) mice. Neonatal mice were injected in the temporal face vein with 1e11 vg of AAV9. Three weeks post-injection, immunolabeling of GFP and HuD revealed robust transduction within myenteric neurons. GFP expression was limited to myenteric neurons and no expression was seen in S100 positive enteric glia. GFP was detected in all sections of the gastrointestinal tract, including the stomach, duodenum, jejunum, ileum, cecum and colon (**Figure 3**).

Figure 4.



Green fluorescent protein (GFP) expression in the myenteric plexus following intravenous injection of AAV9 expressing GFP into juvenile mice. Intravenous injection of AAV9 into P21 mice resulted in robust GFP (green, **A-F**) expression in neurons (HuD, red, **G-L**) in the stomach (**A**, **G**, **M**), duodenum (**B**, **H**, **N**), jejunum (**C**, **I**, **O**), ileum (**D**, **J**, **P**), cecum (**E**, **K**, **Q**), and in the colon (**F**, **L**, **R**). Arrows in merged images (**M-R**) point to GFP expressing neurons. Arrowheads identify untransduced neurons. Scale bars are 100μm.

Juvenile mice were injected in the tail vein with 2e12 vg of AAV9 on P21. As with the neonatal injected mice, immunolabeling of GFP and HuD revealed robust transduction within myenteric neurons throughout all regions of the gastrointestinal tract (**Figure 4**). GFP expression

Figure 5.

Age	Region	Total Ganglia Counted	Total HuD+ Neurons	Total GFP+ Neurons	N	Range of GFP+ cells/animal	Average % Transduction (± SEM)
P1	Stomach	20	869	334	3	52-203	38.6 ± 1.85%
	Duodenum	20	263	77	3	49-84	31.5 ± 3.94%
	Jejunum	20	595	177	3	47-63	29.6 ± 1.56%
	Ileum	20	585	242	3	120-122	41.3 ± 1.89%
	Cecum	20	494	129	3	68-129	25.6 ± 2.04%
	Colon	150	5529	2416	3	631-980	43.7 ± 2.69%
P21	Stomach	20	582	294	3	82-114	51.5 ± 3.95%
	Duodenum	10	266	91	3	21-42	38.8 ±4.82%
	Jejunum	20	487	206	3	47-50	42.6 ±3.38%
	Ileum	37	923	531	3	179-223	57.2 ± 2.56%
	Cecum	35	874	386	3	149-237	43.7 ± 2.15%
	Colon	152	4646	2187	3	598-861	47.1 ± 3.53%

Quantification of GFP expressing myenteric neurons. Total transduced and untransduced neuron counts in each gastrointestinal region in mice injected either at P1 (neonates, n = 3) or P21 (juveniles, n = 3). Transduction of HuD positive neurons ranged from ~25% to ~43% in neonatally injected mice. Transduction ranged from ~42% to 57% in juvenile injected mice.

was again limited to myenteric neurons and no expression occurred in enteric glia. These data show that there is no cell type transduction bias (neurons versus glia) dependent on age of administration within the myenteric plexus of the ENS. Next, GFP expressing myenteric neurons in neonatal and juvenile injected mice (n = 3 in each group) were quantified (**Figure 5**).

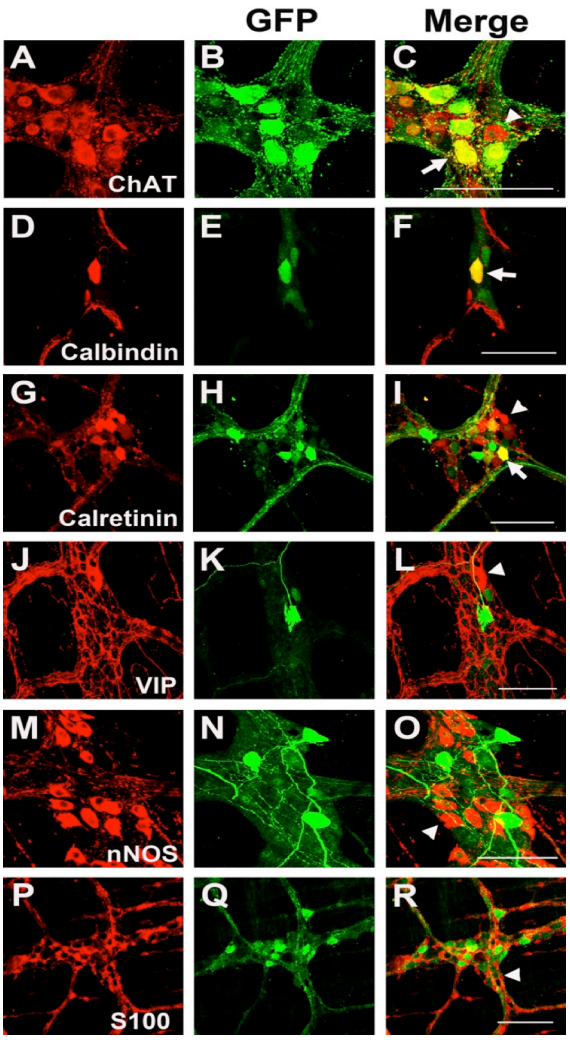
In neonatal mice, transduction ranged from ~25% to ~43% depending on the GI examined. The highest transduction occurred in the colon with 43.7 ± 2% of HuD+ neurons being GFP+. In juvenile mice, transduction ranged from ~42% to ~57%, with the highest transduction occurring in the ileum with 57.2 ± 3% of HuD+ neurons being GFP+.

Chemical coding of transduced neurons

Myenteric neurons can be classified as motor neurons, interneurons and sensory neurons (Furness et al., 2003). These neurons can be further categorized by their chemical coding, which

is based on the chemical markers present within the cell (Costa et al., 1986; Lomax and Furness, 2000). To determine if AAV9 preferinally transduced certain neuronal subtypes, the myenteric plexus of the colon was immunolabeled for GFP, HuD, and either calbindin, calretinin, ChAT, nNOS, or VIP (**Figure 6**). GFP co-expression occurred in ChAT, calbindin and calretinin positive neurons, and transgene expression did not occur in nNos and VIP positive neurons. ChAT is indicative of excitatory motor neurons that project to circular and longitudinal muscle as well as ascending and descending interneurons (Harrington at el., 2010). Calbindin is commonly expressed in descending interneurons (Costa et al., 1986; Furness, 2000) and calretinin is expressed in ascending interneurons (Bergner et al., 2014). nNOS and VIP are expressed in inhibitory motor neurons that project to circular and longitudinal muscle (Furness,

Figure 6.



Chemical coding of transduced myenteric cells in the colon after systemic GFP expressing AAV9 injection. GFP expression (B, E, H, K, N, Q) was detected in choline acetyltransferase (ChAT, A, C) positive, calbindin positive (D, F), and calretinin positive (G, I) myenteric neurons and some intraganglioninc fibers. ChAT staining is indicative of excitatory motor neurons and ascending and descending projecting interneurons. Calbindin and calretinin calcium binding proteins are indicative of excitatory motor neurons and some classes of interneurons. Transduction was rare or completely absent in vasoactive intestinal peptide (VIP, J, L) positive or neuronal nitric oxide synthase (nNOS, M, O) positive cells. VIP and nNOS staining is associated with inhibitory motor neuron and descending interneurons. GFP did not co-localize with S100 positive glial cells (P, R). Arrows indicate co-expression while arrowheads indicate no GFP expression. Scale bars are 100µm.

2000). These data suggests that AAV9 has a bias towards neurons that are involved in excitatory signaling.

Discussion:

Characterizing AAV transduction in the ENS is important for developing novel GI therapies. This study characterized the transduction efficiency of different AAV serotypes, namely AAV1, 5, 6, 8 and 9. Robust transduction was seen in the ENS after delivery of AAV8 and AAV9. Intravenous AAV9, a viral serotype now being delivered by intravenous injection in a clinical trial (Mendell, J.R., 2014), showed no difference in enteric cell tropism following either neonatal or adult injection. This result is contradictory to what occurs in the CNS following intravenous injection in mice. These data suggest that AAV mediated therapy development is feasible for the gastrointestinal tract. AAV gene therapy has the potential to treat both diseases of the gut alone, such as FGIDs, but may also be important for treated gastrointestinal symptoms associated with classical neurological diseases such as Parkinson's disease. Most applicable to these data presented is the ability to develop therapies for functional gastrointestinal disorders (FGIDs) that are caused by the dysfunction of the ENS and inflammation within GI tract.

FGIDs are the most common GI disorders in the general public with estimates being that 1 in 4 people in the U.S have one of these disorders (Talley, 2008; Parkman and Doma, 2006). FGID's can be separated into two separate subgroups, one having psychological and social features and the second having more somatic dysregulation (Bennett et al., 1998), These features cause common symptoms such as altered bowel habits or stool forms, defecatory dysfunction, abdominal distension/bloating, nausea, early satiety and visceral pain (McLaughlin, 2008).

Symptomatic therapy development has been hindered because of heterogeneity of reported symptoms, no method of targeted delivery, and the limited understanding of the biological basis of FGIDs (McLaughlin, 2008). FGID symptoms are currently treated with pharmacotherapies, dietary and lifestyle changes and no cure is available.

When considering AAV as a novel treatment for diseases of the gut, it is important to pinpoint the involvement of the ENS. Alterations in the ENS such as degeneration and functional impairment of enteric neurons are associated with uncoordinated motor activities that result in altered transit of intestinal content (Di Nardo et al., 2008). For example, Hirschprung's disease (HSCR) occurs as a result of an absence of enteric neurons and ganglia in terminal regions of the gut, leading to tonic contraction of the affected segment, intestinal obstruction and distention of the proximal bowel (Heanue and Pachnis, 2007). HSCR is representative of the main genetic cause of functional intestinal obstruction and occurs in 1:5,000 live births (Amiel et al., 2008). Due to the genetic involvement in HSCR and the dysfunction occurring as a result of impaired enteric neurons, AAV can be seen as a promising treatment for the disease. The only current treatment for HSCR is surgical removal of the aganglionic section of the gut. Although this method is able to remove the affected portion of the gut, there are still life-long GI problems that include chronic constipation and soiling (Moore et al., 1996, Yanchar et al., 1999, Amiel et al. 2008). Thus, incorporating a long-term expression of a transgene within the affected enteric neurons can help relieve these effects and regulate normal functioning within the gut.

The most common gene mutations in HSCR are found within the *RET* gene and are identified in 50% of familial and 15-20% of sporadic HSCR cases (Amiel et al., 2008, Di Nardo et al., 2008). There has been over one hundred *RET* mutations that have been described throughout the gene; these mutations include deletions, insertions, missense, nonsense and splicing mutations (Hofstra

et al., 2000, Attie et al., 1995, Angrrist et al., 1995). *RET* is a transmembrane receptor with a cadherin-like extracellular domain, a cysteine-rich region and an intracellular tyrosine kinase domain (Ceccherini et al., 1993). Deletion of *RET* in the mouse leads to complete intestinal aganglionosis (Schuchardt et al., 1994). Activation of the *RET* receptor occurs when a complex is formed with one of its ligands by dimerization, such ligands are those of glial cell-line-derived neurotrophic factor (GDNF) and its co-receptor GDNF family receptor α1 (GFRα1) (Heanue and Pachnis, 2007). Furthermore, there have been patients that have been identified that carry heterozygous mutations in GDNF (Angrist et al., 1995). This ligand/receptor complex is essential for normal ENS development and maintenance of both central and peripheral neurons (Heanue and Pachnis, 2007, Amiel et al., 2008). By inserting a *RET* transgene within AAV, we can establish a constant expression of non-mutated *RET* within enteric neurons and restore functioning of the ENS and relive the symptoms seen in surgically treated patients.

In summary, it is shown that enteric neurons can effectively be targeted for gene therapy following systemic delivery of AAV8 and AAV9. These data can lay a foundation for the possible treatment of HSCR along with other FGID's and neurological disorders that exhibit GI dysfunction.

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